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Construction of Four Double Gene Substitution Human × Bovine Rotavirus Reassortant Vaccine Candidates: Each Bears Two Outer Capsid Human Rotavirus Genes, One Encoding P Serotype 1A and the Other Encoding G Serotype 1, 2, 3, or 4 Specificity

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Previously, four human × bovine rotavirus reassortant candidate vaccines, each of which derived ten genes from bovine rotavirus UK strain and only the outer capsid protein VP7-gene from human rotavirus strain D (G serotype 1), DS-1 (G serotype 2), P (G serotype 3), or ST3 (G serotype 4), were developed [Midthun et al., (1985): Journal of Virology 53:949–954; (1986): Journal of Clinical Microbiology 24:822–826]. Such human × bovine reassortant vaccines should theoretically provide antigenic coverage for the four epidemiologically most important VP7(G) serotypes 1, 2, 3, and 4. In an attempt to increase the antigenicity of VP7-based human × animal reassortant rotavirus vaccines which derive a single VP7-encoding gene from the human strain and the remaining ten genes from the animal strain, we generated double gene substitution reassortants. This was done by incorporating another protective antigen (VP4) of an epidemiologically important human rotavirus by crossing human rotavirus Wa strain (P serotype 1A), with each of the human × bovine single VP7-gene substitution rotavirus reassortants. In this way four separate double gene substitution rotavirus reassortants were generated. Each of these reassortants bears the VP4-encoding gene from human rotavirus Wa strain, the VP7-encoding gene from human rotavirus strain D, DS-1, P, or ST3, and the remaining nine genes from bovine rotavirus strain UK. The safety, antigenicity, and protective efficacy of individual components as well as combinations of strains are currently under evaluation. ***J. Med. Virol. 51:319–325, 1997.***

KEY WORDS: double gene substitution rotavirus reassortants; rotavirus reassortant vaccines; rotavirus VP4 and VP7

INTRODUCTION

Diarrheal diseases are a leading cause of death in infants and young children in developing countries. Because group A rotaviruses are consistently the single most important group of etiologic agents of severe diarrhea in infants and young children under 2 years of age in both developed and developing countries [Bern and Glass, 1994; Kapikian and Chanock, 1996], the development of a rotavirus vaccine is an important global public health goal supported by various groups including the World Health Organization [Bishop, 1993; Conner et al., 1994; Glass et al., 1994; Hoshino and Kapikian, 1994b; Programme for Control of Diarrhoeal Diseases, 1994].

Initially, the "Jennerian" approach, in which bovine or simian rotaviruses were developed as attenuated live oral vaccines for humans, was pursued by various groups [Clark et al., 1986a; Kapikian et al., 1985; Vesikari et al., 1983; Wyatt et al., 1985]. The protective efficacy of such monovalent vaccines was variable [Georges-Courbot et al., 1991; Kapikian, 1994; Kapikian et al., 1992; Vesikari, 1993, 1994], especially in unprimed infants less than 6 months of age who characteristically developed a homotypic serologic response to the VP7(G) serotype of the vaccine virus but more often failed to develop a serologic response to other rotavirus serotypes of epidemiological importance [Green et al., 1990]. Since the distribution of the four epidemiologically important serotypes in the community is unpredictable, the monovalent "Jennerian" approach was modified in order to achieve broader antigenic coverage. Thus, reassortants with a single gene derived from a human rotavirus that encodes outer capsid VP7 with G1, G2, or G4 specificity and the re-

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maining genes derived from rhesus monkey rotavirus (RRV) MMU18006 were combined into a quadrivalent vaccine that included RRV as the G3 component. A similar approach was taken with the bovine rotavirus UK strain except single gene substitution reassortants were generated for each of the four serotypes because the UK strain is a G6 strain [Midthun et al., 1985, 1986]. The human × RRV quadrivalent vaccine is undergoing trials in various parts of the world with promising results [reviewed in Bernstein et al., 1996; Kapikian, 1994; Kapikian et al., 1992; Rennels et al., 1996].

Although each of the six proteins comprising the mature group A rotavirus particle can induce its specific antibodies [Estes and Cohen, 1989; Hoshino and Kapikian, 1994c; Mattion et al., 1994], only antibodies directed against the two outer capsid proteins (VP4 and VP7) are neutralizing [Greenberg et al., 1983; Hoshino et al., 1985; Offit and Blavat, 1986b]. Furthermore, although protection against rotavirus disease appears to be multifactorial [reviewed in Offit, 1994], studies in experimental animals indicated that VP4 and VP7 are independent protective antigens and antibodies to either protein can confer resistance to virulent rotavirus [Hoshino et al., 1988; Matsui et al., 1989; Offit et al., 1986a]. In addition, recent studies indicate that the four epidemiologically most important human rotavirus VP7 serotypes share a common VP4 serotype that includes two related subtypes designated P1A or P1B (G1 P1A, G2 P1B, G3 P1A, and G4 P1A) [Gentsch et al., 1992; Gunasena et al., 1993; Larralde and Flores, 1990; Mphahlele and Steele, 1995; Santos et al., 1994; Silverstein et al., 1995; Steele et al., 1993, 1995; Timenetsky et al., 1994; Ushijima et al., 1994]. Although P serotypes 1A and 1B, which belong to different genotypes [8] and [4], respectively [Estes, 1996], were initially reported to share a one-way cross neutralization relationship [Gorziglia et al., 1990], recent data obtained from analyses of hyperimmune antisera raised against various single gene substitution reassortants [Hoshino and Kapikian, 1996a], and of infection sera obtained from neonates who underwent natural primary rotavirus infection [Flores et al., 1989; Offit et al., 1993; Rojas et al., 1995] appears to suggest that the P1A and P1B are two distinct serotypes. The serotype that includes P1A and P1B is distinct from the VP4 serotype of the simian or bovine rotavirus included in vaccines currently in clinical trial [Gorziglia et al., 1990; Hoshino and Kapikian, 1996b; Mackow et al., 1990].

In an attempt to maximize immunogenicity of live rotavirus candidate vaccines and utilize not only the VP7 protective antigen but also the VP4 protective antigen of epidemiologically important human rotaviruses, we recently selected a series of cold-adapted (*ca*) and temperature-sensitive (*ts*) mutants of human rotaviruses of epidemiologic importance [Hoshino et al., 1994a]. These strains are currently under evaluation in phase I clinical trials. In the present study we pursued another approach to increasing immunogenicity of live rotavirus vaccine by broadening the "modified Jennerian" approach. Four human × bovine rotavirus reassortants were selected that contained not only a VP7 with G1, G2, G3, or G4 antigenic specificity but also a VP4 of P1A antigenic specificity, the major VP4 of epidemiological significance.

MATERIALS AND METHODS

Viruses, Cell Cultures, Culture Medium, and Hyperimmune Antisera

An experimental vaccine suspension containing human rotavirus Wa (G1) strain (Lot HRV-5) was triply plaque purified in primary African green monkey kidney (AGMK) cells. Generation and characterization of four different human × bovine rotavirus reassortants each with a single VP7-encoding gene derived from a human rotavirus and the remaining ten genes derived from bovine rotavirus UK strain were reported previously [Midthun et al., 1985, 1986]. These single gene substitution reassortants were designated UK × D (G1); UK × DS-1 (G2); UK × P (G3); and UK × ST3 (G4). Experimental vaccine suspensions containing UK × D(Lot HDBRV-I), UK × DS-1 (Lot HDS1BRV-1), UK × P (Lot HPBRV-2), or UK × ST3 (Lot HST3BRV-2) that had been prepared in fetal rhesus monkey lung diploid cell strain (FRhL-2) were used in the attempts to achieve further genetic reassortment described in this study. The human and animal rotaviruses with various combinations of VP7 G and VP4 P antigenic specificities used for characterization of antibody response to double gene substitution human × bovine rotavirus reassortants are listed in Table I and have been described previously [Clark et al., 1987; Gerna et al., 1985; Matsuno et al., 1985; Stuker et al., 1980; Urasawa et al, 1982, 1990; Woode et al., 1975; Wyatt et al., 1982, 1983].

Primary cultures of AGMK cells (Whittaker Bioproducts, Walkersville, MD) were used for genetic reassortment, plaque purification, and virus amplification. The established monkey kidney MA104 cell line was used for virus titration and plaque reduction neutralization (PRN) assay. Eagle's minimum essential medium supplemented with 0.5 μg /ml trypsin(SIGMA Type IX, St. Louis, MO) and antibiotics was used as maintenance medium. Hyperimmune antiserum to each of the double gene substitution rotavirus reassortants was raised in specific-pathogen-free guinea pigs (National Cancer Institute, Frederick, MD) which were free of rotavirus neutralizing antibodies (titer <1:20) as determined by PRN assay. Rotavirus immunogens were prepared as previously reported [Wyatt et al., 1982]

Generation and Characterization of Double Gene Substitution Rotavirus Reassortants

Primary roller tube cultures of AGMK cells were coinfected at a multiplicity of infection of approximately one with the Wa virus and the UK × D, UK × DS-1, UK × P, or UK × ST3 reassortant virus. When approximately 75% of the cells exhibited cytopathic effects, the cultures were frozen and thawed once and the

TABLE I. Antigenic Characterization of Double Gene Substitution Reassortant Rotaviruses as Determined by PRN Assay

				Reciprocal of 60% PRN antibody titer of guinea pig hyperimmune antiserum to indicated rotavirus							
Rota virus strain	Species of origin	G Type	P Type	Wax (Dx UK) (P1A, G1)	Wa (P1A, G1)	Wax (DS-1 x UK) (P1A, G2)	DS-1 (P1B,G2)	Wax (Px UK) (P1A, G3)	P (P1A, G3)	Wax (ST3 x UK) (P1A,G4)	ST3 (P2,G4)
Wa	human	1	1A	*10,240**	*81,920*	2,560	<80	2,560	80	2,560	80
K8	human	1	3	*10,240*	*20,480*	640	<80	<80	<80	<80	<80
DS-1	human	2	1B	160	<80	*10,240*	*10,240*	320	<80	320	<80
P	human	3	1A	320	640	640	<80	*40,960*	*40,960*	640	80
MMU 18006	rhesus monkey	3	5	160	<80	160	<80	*20,480*	*40,960*	160	<80
ST3	human	4	2	160	80	160	<80	160	<80	*40,960*	*40,960*
VA70	human	4	1A	640	640	1,280	<80	1,280	80	*20,480*	*20,480*
UK	bovine	6	7	<80	<80	<80	<80	80	<80	<80	<80
69M	human	8	4	<80	<80	<80	<80	640	320	<80	<80
WI61	human	9	1A	160	320	640	<80	20,480	80	2,560	<80
L26	human	12	1B	160	80	640	160	2,560	80	640	<80
Wax(Dx UK)	reassortant	1	1A	*20,480*	ND	ND	ND	ND	ND	ND	ND
Wax (DS-1 x UK)	reassortant	2	1A	ND	ND	*20,480*	ND	ND	ND	ND	ND
Wax(Px UK)	reassortant	3	1A	ND	ND	ND	ND	*81,920*	ND	ND	ND
Wax (ST3 x UK)	reassortant	4	1A	ND	ND	ND	ND	ND	ND	*81,920*	ND

*VP7-homologous values are italicized. ND = Not done.

lysate was plated on primary AGMK cells in a six-well plate in the presence of G1-specific VP7 neutralizing monoclonal antibody 2C9 [Shaw et al., 1985] for selection of the desired DS-1, P or ST3 VP7 gene reassortant. Reassortant Wa × (D × UK) was identified without such immunological pressure because Wa and D share the same VP7 serotype. The desired reassortants were selected and identified or only identified in the case of Wa × (D × UK) and then plaque purified three times. One hundred and forty-four, 180, 540, or 108 plaques were analyzed in order to identify the desired Wa (P1A) × (D (G1) × UK), Wa × (DS-1 (G2) × UK), Wa × (P (G3) × UK), or Wa × (ST3 (G4) × UK) double gene substitution reassortants. Each plaque purified reassortant was examined for serotype by enzyme-linked immunosorbent assay using type-specific VP7 neutralizing monoclonal antibodies (mAbs) designated as follows: KU-4 [Taniguchi et al., 1987], 2C9, and 5E8 [Padilla-Noriega et al., 1990] (G1-specific); S2-2G10 [Taniguchi et al., 1987] (G2-specific); 954/159/13 [Greenberg et al., 1983], YO-1E2 [Taniguchi et al., 1987] (G3-specific); and ST-2G7 [Taniguchi et al., 1987] (G4-specific). The origin of the genes of each reassortant was identified by polyacrylamide gel electrophoresis (PAGE) of its genomic RNAs. Hyperimmune guinea pig antiserum to each reassortant was tested for antibodies to selected human and animal rotaviruses by 60% plaque reduction neutralization (PRN) assay.

RESULTS

The lysate of cells coinfected with Wa (P1A;G1) virus and a single VP7-gene substitution human × bovine reassortant rotavirus containing a single human rotavirus gene that encodes the VP7 of strain D, DS-1, P, or ST3 was plated onto primary AGMK cells. Four different double gene substitution reassortant rotaviruses were identified. Each of these reassortants derived its VP4-gene from human rotavirus Wa (P1A), its VP7-gene from human rotavirus D(G1), DS-1(G2), P(G3), or ST3(G4) strain, and the remaining nine genes from bovine rotavirus UK strain as confirmed by PAGE analysis (Figs. 1 and 2). When tested in an enzyme-linked immunosorbent assay, each plaque purified double gene substitution reassortant and the corresponding parental single VP7-gene substitution reassortant reacted similarly with a collection of seven distinct serotype-specific VP7 neutralizing monoclonal antibodies (data not shown), suggesting that the fine structure and distribution of neutralization epitopes on VP7 remained intact. Reassortants selected under immunologic pressure exerted by VP7 G1 monoclonal antibody 2C9 were confirmed by PRN to exhibit the predicted G2, G3, or G4 VP7 serotype using the appropriate monoclonal antibodies (data not shown).

Guinea pigs that were hyperimmunized with the P1A G1, P1A G2, P1A G3, or P1A G4 double gene substitution reassortant rotavirus developed high levels of VP7-homotypic and moderately high levels of VP4-homotypic neutralizing antibodies as well as a varying degree of heterotypic neutralizing antibodies (Table I). Hyperimmune antiserum raised to Wa × P × UK reassortant neutralized not only VP7-homotypic strain P and VP4-homotypic strains Wa, VA70, and WI61, but also both VP7- and VP4-heterotypic strains 69M and L26. This observation may reflect the fact that G3 rotaviruses share a high degree VP7 amino acid homol-

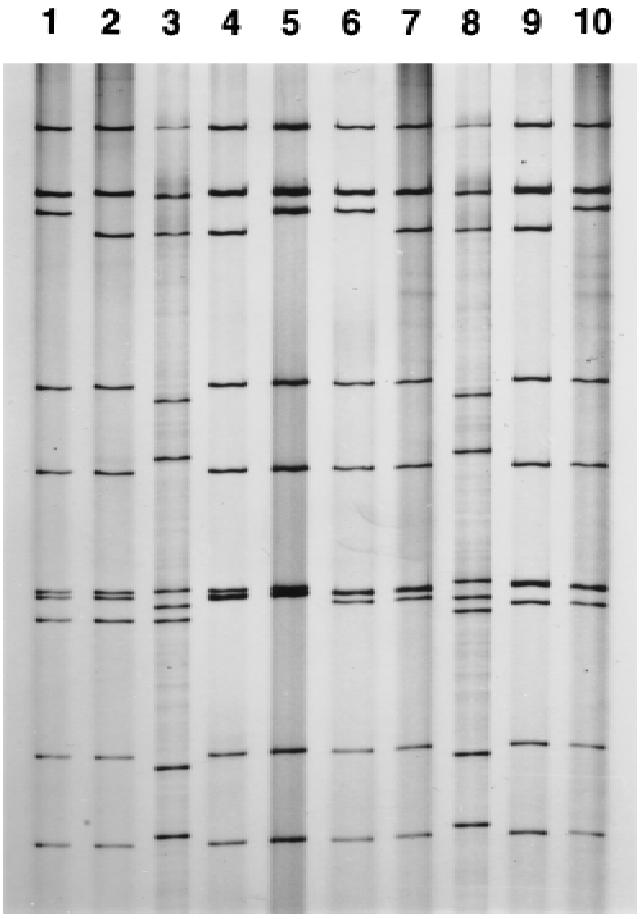

Fig. 1. Electrophoretic migration patterns of genomic RNAs of UK × D reassortant (**lane 1**), Wa × (D × UK) reassortant (**lane 2**), Wa strain (**lane 3**), Wa × (DS-1 × UK) reassortant (**lane 4**), UK × DS-1 reassortant (**lane 5**), UK × P reassortant (**lane 6**), Wa × (P × UK) reassortant (**lane 7**), Wa strain (**lane 8**), Wa × (ST3 × UK) reassortant (**lane 9**), and UK × ST3 reassortant (**lane 10**). Genomic RNAs were electrophoresed in 15% polyacrylamide gel at 20 mA for 15 hr, and the resulting migration patterns were visualized by staining of gel with silver nitrate.

ogy in one of three variable regions involving neutralization (amino acids 208 to 221) with G8 (69M strain), G9 (WI61 strain) and G12 (L26 strain) [Browning et al., 1991]. The contribution of the VP4 P1A of the double gene reassortant viruses to the induction of homologous neutralizing antibodies can be estimated by comparing the neutralization titer against viruses with the same VP4 P1A and a VP7 G serotype different from the immunizing double gene substitution reassortant. These comparisons made with data from Table I suggest that the VP4 P1A induced significant amounts of neutralizing antibodies but VP7 G1, 3 or 4 stimulated considerably more neutralizing activity (Table I). Of note is the finding that the magnitude of the VP4-homotypic neutralizing antibodies induced by the double gene substitution reassortant viruses was greater than that of hyperimmune antiserum to reference cell culture human rotavirus strains such as Wa, DS-1, P, or ST3. Nine of 11 genes of each double gene substitution reassortant were derived from the bovine

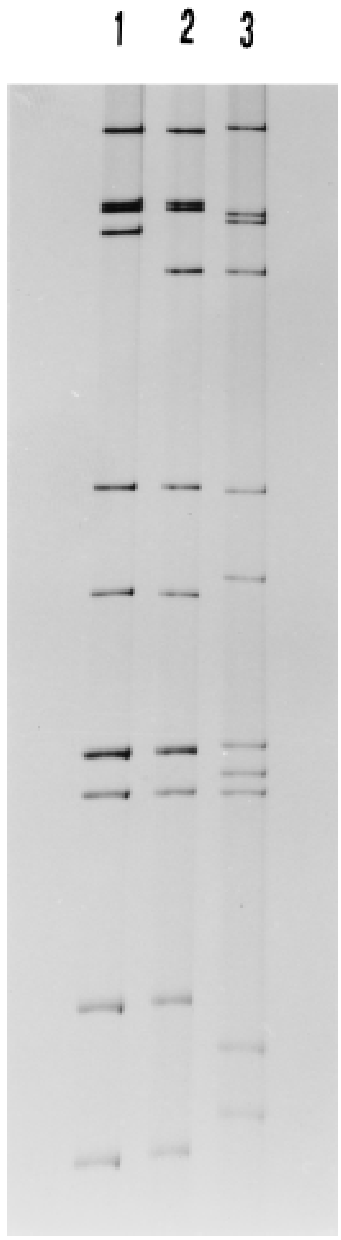

Fig. 2. Electrophoretic migration pattern of genomic RNAs of UK × D reassortant (**lane 1**), Wa × (D × UK) reassortant (**lane 2**), and Wa strain (**lane 3**). Genomic RNAs were electrophoresed in 15% polyacrylamide long gel at 15mA for 24 hr, and the resulting migration patterns were visualized by staining gel with silver nitrate.

rotavirus UK strain but were not coding for neutralization specific antigens. Therefore, hyperimmune antisera raised against the double gene substitution reassortants failed to show significant neutralizing activity against the bovine rotavirus.

DISCUSSION

Currently most live oral rotavirus reassortant vaccines, which are in various stages of evaluation for protective efficacy in various parts of the world, are outer

capsid protein VP7-based vaccines. In an attempt to broaden the immunologic potential of live vaccine strains while retaining the attenuation phenotype, we modified existing human × animal rotavirus reassortants and generated new reassortant candidate vaccines carrying both VP4 and VP7 protective antigens of epidemiologically most important human rotavirus serotypes, *i.e.,* VP4 1A, and VP7 G1, G2, G3, and G4.

Bovine rotavirus vaccines based on the "Jennerian" approach have been evaluated extensively in human volunteers [reviewed in Kapikian et al., 1992; Vesikari, 1994]. Such live oral bovine rotavirus vaccines including NCDV strain (RIT4238), WC3 strain, and UK strain have been shown to be highly attenuated in susceptible humans. Furthermore, clinical evaluation of live oral bovine × human rotavirus reassortant vaccines such as WC3 × human rotavirus reassortant [Clark et al., 1986b] and UK × human rotavirus reassortant vaccines has demonstrated that the bovine rotaviruses function effectively as donors of genes that confer the attenuation phenotype on virulent wild-type human rotaviruses. In this study we chose bovine rotavirus UK strain as a donor of genes that confer the attenuation phenotype.

We chose human rotavirus Wa strain (P1A, G1) as a VP4-gene donor because: (i) the majority of the human rotavirus field isolates of epidemiologic importance analyzed in various parts of the world have been shown to carry VP4(P) serotype 1A specificity, and (ii) P serotype 1A VP4 capsid protein has been demonstrated to induce broadly cross-reactive neutralizing antibodies after parenteral immunization [Gorziglia et al., 1990], or natural primary infection [Flores et al., 1989; Offit et al., 1993; Rojas et al., 1995].

In a recent study involving a virulent porcine rotavirus, an avirulent human rotavirus, and reassortants therefrom we observed that four genes, i.e., those encoding VP3, VP4, VP7, and NSP4[NS28], each play an important independent role in virulence of the porcine rotavirus and attenuation of the human rotavirus for colostrum-deprived gnotobiotic piglets [Hoshino et al., 1995]. We also showed that only a four gene substitution reassortant, which contained VP3, VP4, VP7, and NSP4 genes of a virulent porcine rotavirus in the genetic background of the avirulent human rotavirus, restored virulence. Hence, although each of the double gene substitution rotavirus reassortants generated in this study carries both the VP4- and the VP7-encoding genes from virulent human rotaviruses, each also carries the VP3- and NSP4(NS28)-encoding genes from the attenuated bovine rotavirus strain, making it unlikely that these reassortants will be virulent in humans.

We and others have previously noticed that the antigenicity and immunogenicity of one or both outer capsid proteins of a reassortant is often slightly different from that of the corresponding parental rotaviruses [reviewed in Gombold and Ramig, 1994]. It was of interest, therefore, that the VP4 outer capsid protein of the double gene substitution rotavirus reassortants appeared to be more immunogenic than that of the parental rotavirus. Whether this enhanced immunogenicity results in the induction of broadly cross-reactive neutralizing antibodies after oral immunization requires evaluation in unprimed individuals.

The formulation and delivery of rotavirus vaccines requires a knowledge of the diversity and distribution of rotavirus VP7 and VP4 serotypes circulating in a community. Hence, epidemiologic surveys of rotavirus G and P types has been carried out in various parts of the world [Beards et al., 1989; Bern et al., 1992; Birch et al., 1988; Flores et al., 1988; Gentsch et al., 1992; Gunasena et al., 1993; Larralde and Flores, 1990; Mphahlele and Steele, 1995; Nakagomi et al., 1990; Padilla-Noriega et al., 1990; Santos et al., 1994; Silverstein et al., 1995; Steele et al., 1993, 1995; Timenetsky et al., 1994; Unicomb et al., 1989; Urasawa et al., 1989; Ushijima et al., 1994]. Such studies have shown consistently that the majority of typeable human rotaviruses of epidemiologic importance carry P serotype 1A or 1B and G serotype 1, 2, 3, or 4 specificities. Because: (i) the immune response after oral rotavirus vaccination of an unprimed individual is primarily homotypic, and (ii) rotavirus vaccine efficacy might be increased by providing antigenic coverage for both the VP4 and VP7 of epidemiologically important rotavirus serotypes, we generated double gene substitution rotavirus reassortants. Collectively these reassortants display both VP4(P1A) and VP7(G1, G2, G3, or G4) protective antigens of the human rotaviruses currently recognized as being most important epidemiologically. Safety, immunogenicity, as well as protective efficacy of these reassortant rotavirus candidate vaccines will be evaluated, singly or in combination of two or more, in susceptible humans.

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